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Heterogeneity of Hepatitis C Virus Genotypes in Hemophilia: Relationship With Chronic Liver Disease

By F.E. Preston, L.M. Jarvis, M. Makris, L. Philp, J.C.E. Underwood, C.A. Ludlam, and P. Simmonds

In this study we have determined the hepatitis C virus (HCV) serotype and genotype in a cohort of 96 HCV-infected hemophiliacs and have examined the relationship between HCV genotype and severity of chronic liver disease as determined by liver biopsy. HCV serotype was determined by specific enzyme-linked immunosorbent assays (ELISAs) and genotype by restriction fragment length polymorphism (RFLP) and HCV viral sequencing. The pattern of genotype distribution was quite unlike that of HCV-infected United Kingdom (UK) blood donors in that five of the six known HCV genotypes were represented, 50% were type 1, 13% type 2, and 18% type 3. An unexpected observation was the presence of HCV genotype 4 in four patients and type 5 in two pa-

tients. An additional feature was the presence of mixed infection, detected in 14% and 7% by serotype and genotype analysis, respectively. Liver biopsies were available from 51 patients. Cirrhosis was present in five of 27 (19%) of individuals with type 1, in 2 of 9 (22%) with type 2, and 5 of 8 (63%) of those with type 3. The heterogeneous pattern of HCV genotype distribution in this cohort of patients and the observed relationship between the severity of the related liver disease and specific HCV genotype may have important implications with respect to the natural history and treatment of HCV-related chronic liver disease in infected hemophiliacs worldwide.

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THERE IS NOW general acceptance that the hepatitis C virus (HCV) is the cause of virtually all cases of posttransfusion hepatitis in western countries and is responsible for most of the observed chronic liver disease in treated hemophiliacs. Most of the early reports of hepatic dysfunction in hemophiliacs coincided with the introduction of clotting factor concentrates in the early 1970s,^{1,2} and it is now clear that before the widespread adoption of effective antiviral treatment of these products in the mid 1980's they were largely responsible for the transmission of HCV in the hemophilia population. This view receives support from the observation that virtually 100% of treated hemophiliacs are HCV antibody positive by second generation assays.³

HCV has been classified into a number of distinct groups or genotypes on the basis of sequence similarities of complete genomic sequences⁴ or fragments amplified by polymerase chain reaction (PCR).^{5,6} All known variants can be classified into a total of six major genotypes (types 1 through 6), each with a variable number of more closely related subtypes.⁷ The six major genotypes have distinct nucleotide sequence in the 5' noncoding region (NCR).^{8,9} Therefore, viruses can be typed by restriction fragment length polymorphism (RFLP) analysis of DNA amplified from this region of the HCV genome.¹⁰

Single batches of clotting factor concentrates are derived from tens of thousands of donor units and regularly treated patients will have been exposed to high concentrations of virus. In addition, it is likely that mixed infection will be a feature of HCV infection hemophilia.

In this study, we have used (1) RFLP, (2) specific ELISAs using synthetic HCV viral peptides, and (3) HCV viral sequencing to determine the HCV genotypes of 96 HCV-infected hemophiliacs. We have also attempted to relate viral genotype to the severity of chronic liver disease determined by liver biopsy, clinical progression, and liver enzyme determination.

MATERIALS AND METHODS

Our cohort comprises 96 hepatitis C positive patients with familial hemorrhagic disorders treated in Sheffield, UK. All but four received nonvirally inactivated concentrates during the period 1972 to 1985. The other four patients received heat-treated products clearly implicated in HCV transmission.¹¹ Patients with hemophilia B received nonvirally inactivated prothrombin complex concentrates derived

from volunteer UK donors only, whereas the remainder of the cohort received both commercial and noncommercial factor concentrates and in the latter group, those patients on regular treatment were exposed to a variety of different products.

All subjects have been followed prospectively. Patients with severe hemophilia (A and B) were reviewed at least three times a year, while the others (including those with von Willebrand's disease) were reviewed annually. Liver enzyme determination was performed at each visit. Alanine transferase (ALT) concentrations were considered persistently raised when all three most recent measurements were high, intermittently raised when one or two of the last three values were high, or normal when none of the last three estimations was above the upper limit of the normal range.

Liver biopsy samples were available from 51 of the 96 patients in the study. For those patients who had more than one biopsy, only the most recent one is considered. Forty-one biopsies were obtained as a diagnostic procedure, and 10 were obtained at autopsy. All were reviewed and categorized histologically by one pathologist (J.C.E.U.).

RNA Extraction

Virus RNA was extracted from 0.5 mL of stored sera after pelleting of the virus by centrifugation at 100,000g for 90 minutes at 4°C, incubation at 37°C for 2 hours with 1 mg/mL proteinase K in the presence of 40 µg/mL polyadenylic acid, 0.5% sodium dodecyl sulphate, 0.1 mol/L NaCl, 50 mmol/L Tris HCl (pH 8.0), and 1 mmol/L EDTA. RNA was extracted with phenol, and after centrifu-

From the University Department of Haematology, Royal Hallamshire Hospital, and the Department of Pathology, University of Sheffield Medical School, Sheffield, UK; and the Department of Medical Microbiology, University of Edinburgh, Medical School, Edinburgh, and the Edinburgh Haemophilia Centre, Royal Infirmary, Edinburgh, UK.

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Address reprint requests to F.E. Preston, MD, University Department of Haematology, Royal Hallamshire Hospital, Glossop Rd, Sheffield, UK S10 2JF.

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gation the supernatant was re-extracted successively with phenol/chloroform (1:1) and chloroform/isoamylalcohol (50:1). Nucleic acid was precipitated by the addition of 0.1 vol of sodium acetate (pH 5.2) and 2 vol of ethanol. The dried pellet was resuspended in 25 μ L of diethylpyrocarbonate (DEPC)-treated water.

HCV Typing

RNA was reverse-transcribed and amplified using nested primers matching conserved regions in the 5' NCR as previously described.⁵ Amplification of HCV RNA was performed using outer primers 209 and 939 for the primary PCR and inner primers 211 and 940 for secondary PCR.⁵ Twenty-five-microliter aliquots of product DNA were cleaved with the restriction enzymes *HaeIII/RsaI* and *MvaI/HinfI*,¹⁰ and the fragments separated by agarose gel electrophoresis using 4% Metaphor agarose (FMC Bioproducts, Rockland, ME). The cleavage patterns in the 5' NCR amplified by PCR varied considerably between the major types of HCV, and it was possible to identify all six major genotypes with this system.

Direct nucleotide sequencing of PCR products was performed as described⁵ with the exception that DNA was purified using Magic PCR preps DNA purification systems (Promega, Madison, WI).

Serum samples were tested for type-specific antibody by an indirect ELISA¹² with the modification that the sample was added at a 1/10 dilution to antigen-coated and blocked wells, and the first incubation carried out at 4°C overnight.

Statistics

Fisher's exact test with Yates' continuity correction was used to test differences between groups.

RESULTS

Liver Function Tests

Persistently abnormal ALT levels were observed in 56% of HCV genotype 1 patients, 75% of type 2, and 88% of type 3. Intermittently abnormal results were seen in 27% of type 1, 25% of type 2, and in only one patient with HCV genotype 3. Normal ALT levels were observed in 17% of type 1 subjects, in a single patient only with type 3, and in three of six PCR negative patients. Of the other three PCR negative subjects, persistently abnormal liver enzymes were seen in two, and intermittent abnormalities in one.

Liver Histology

Biopsy specimens from 28 patients were classified as chronic persistent hepatitis, 10 as chronic active hepatitis, and 13 as cirrhosis. One patient who was PCR negative had chronic persistent hepatitis.

HCV Serotypes

Results of the HCV typing studies are presented in Table 1. Of the 96 hemophiliacs tested, 59% were serotype 1, 2% type 2, and 5% type 3. Mixed serotypes were demonstrated in 14%. In 19 cases (20%), the serotype could not be determined by ELISA.

HCV Genotypes

As indicated in Table 1, there was considerable discordance between individual genotype and serotype, and identical results were observed in 44 of the 77 individuals in whom it was possible to serotype. On genotype analysis,

Table 1. HCV Types in Hemophiliacs as Detected by ELISA Serotypes and RFLP Genotypes

		Serotype				
		1	2	3	Mixed	Nonspecific
Genotype	1	36	0	0	7	5
	2	5	2	0	3	2
	3	6	0	5	2	4
	4	2	0	0	0	2
	5	2	0	0	0	0
	Mixed	3	0	0	1	3
	PCR neg	3	0	0	0	3

The ELISA does not detect HCV types 4 or 5.

50% were type 1, 13% type 2, and 18% type 3. In addition, four patients were type 4 and two type 5. Seven patients had a mixed infection by this method (Table 1). Mixed infections and HCV genotypes 4 and 5 were observed only in patients who had received commercially derived products.

We also examined the relationship between HCV genotype and human immunodeficiency virus (HIV) antibody status. The incidence of coinfection with HIV among the three HCV genotypes 1, 2, and 3 was 27%, 25%, and 35%, respectively.

In view of the observed discrepancies between HCV serotype and genotype, HCV virus sequencing was performed on samples from seven such patients. For all seven samples, identical results were obtained in the genotype derived from RFLP analysis and that from direct sequencing.

Relationship Between Liver Histology and HCV Genotype

Cirrhosis was present in 5 of 27 (19%) biopsy specimens of individuals with type 1; in 2 of 9 (22%) individuals with type 2, and in 5 of 8 (63%) with type 3 (Table 2). The incidence of cirrhosis was significantly greater with type 3 compared with non-type 3 HCV (5 of 8 v 8 of 34) [$P < .05$]. In patients with genotype 1, cirrhosis was documented on biopsy at 12, 15, 20, and 21 years following the first exposure to pooled clotting factor concentrates, compared with patients with genotype 3, where it was found after 3, 4, 6, 18, and 20 years. There were no significant differences between the HCV groups with respect to duration of HCV infection from initial exposure, age, or in type or severity of hemostatic defect (results not given). In addition, there was no apparent relationship between coexistent HIV infection and the presence of hepatic cirrhosis. Two patients were coinfecting with the hepatitis B virus; in one the HCV genotype was 1 and histology chronic persistent hepatitis (CPH), while in the other the HCV genotype was 2 + 3 and the histology chronic active hepatitis (CAH). In one patient with genotype 2 HCV, alcohol abuse was believed to have played a contributory role in the development of cirrhosis.

DISCUSSION

The transmission of HCV to hemophiliacs by clotting factor concentrates in the period before the introduction of effective viral inactivation procedures is well established. This occurred largely as a consequence of the very large donor

Table 2. HCV Genotypes in Relation to Liver Histology

HCV Genotype	Histology		
	CPH No. (%) [*]	CAH No. (%)	Cirrhosis No. (%)
1	15 (56)	7 (26)	5 (19)
2	4 (44)	3 (33)	2 (23)
3	3 (38)	0	5 (63)
4	2 (66)	0	1 (33)
5	1	0	0
1 + 2	1	0	0
1 + 3	1	0	0
Negative PCR	1	0	0

^{*} Percentage within each genotype.

pool incorporated into individual batches of the concentrate. The infectivity of the final material would also relate to the prevalence of HCV in the donor population. Because the prevalence of HCV among paid donors was greater than that among volunteer donors, it can be anticipated that the infectivity of commercial products derived from the former was greater than that of material obtained from the latter group. However, Kernoff et al¹³ and Fletcher et al¹⁴ showed that the infectivity of National Health Service (volunteer) clotting factor concentrates given to previously untreated hemophiliacs was virtually 100%.

There is now considerable evidence that hepatitis C, a single-stranded RNA virus, exhibits considerable sequence diversity, and recent phylogenetic analysis of the viral genome indicates the presence at least six major genotypes of the virus.⁷ Studies from blood donors and from patients with non-A, non-B hepatitis indicate a widespread geographic distribution of HCV types 1 and 2. HCV type 3 has been frequently reported in the United States, Canada, Australia, Europe^{5,15,16} and Thailand.¹⁷

With respect to hemophilia, the geographic distribution of the virus and possible type-related differences in the natural history of the associated chronic liver disease are of considerable interest because the therapeutic materials are derived from donors throughout the world. Consequently, it can be anticipated that the distribution of HCV types in infected hemophiliacs will relate directly to the source of their therapeutic materials.

The cohort of patients reported here have received a range of clotting factor concentrates derived from both volunteer and paid donors. The former are UK-based and the latter are largely from the United States. The distribution of HCV genotypes 1, 2, and 3 were 50%, 13%, and 18%, respectively. In addition, mixed infections were identified by both serotypic (14%) and genotypic analysis (7%). This HCV genotype distribution is markedly different from that of a recently described UK blood donor population in which mixed infections were not observed.¹⁰ Interestingly, in the patients reported here, mixed infections were confined to those who had received commercially derived products.

An unexpected observation, confirmed by sequencing analysis, was the identification of HCV genotype 4 in four of our patients and genotype 5 in a further two. To date, both of these HCV genotypes show highly restricted geographic

distributions in that type 4 has been reported from the Middle East and Zaire,^{7,18} and type 5 occurs predominantly in South Africa,⁶ although recent reports indicate that it is present in low frequency in The Netherlands,¹⁹ Australia,¹⁰ and Canada.¹⁶ Taken together, these two HCV genotypes comprise 6.3% of the infective HCV agent in our patients. Therefore, it seems highly likely that this is a reflection of the source of the donor pool. Because our patients have received a number of different clotting factor concentrates, we are unable to relate the transmission of these HCV genotypes to any particular product. However, infection with HCV genotypes 4 and 5 was confined to patients with hemophilia A. Although the numbers are small, it is possible that commercial products were responsible for the transmission of these genotypes because hemophilia B patients were treated exclusively with UK-derived material, and these particular genotypes have not been identified in UK donors.

In this cohort of hemophiliacs, some differences were observed between HCV serotype and genotype. This is in contrast to the situation in HCV-infected blood donors in whom there is close agreement between the two assays. The observed differences in hemophiliacs is likely to relate to their reinfection with different HCV types, which could differ in their replication rates and in their capacity to induce HCV antibody formation. The recent observation that changes in the major circulating HCV genotype can occur in some infected hemophiliacs (Simmonds, unpublished observation) may also provide some explanation for the observed discrepancies between HCV serotype and genotype in our patients.

Simmonds et al⁷ have suggested that the degree of sequence variability of HCV is sufficient to significantly alter the antigenic and biologic properties of the virus, and they have demonstrated that in HCV antibody positive Scottish blood donors alanine aminotransferase levels are greater in individuals infected with HCV type 3 than in those with the more common type 1 infection. Other groups have also reported possible differences in the natural history of different HCV serotypes and also in their responsiveness to interferon,²⁰⁻²² but the adoption of different HCV classification systems by the various groups makes comparisons somewhat difficult.

We have confirmed previous suggestions that HCV genotype 3 is associated with a more aggressive form of chronic liver disease than that associated with types 1 and 2.¹⁰ Because there were no differences between the HCV genotype groups with respect to duration of exposure, coexistent HIV infection, age, and severity of hemophilia, we conclude that this effect is probably HCV-type specific. It was interesting that in three patients with type 3 HCV cirrhosis developed within 3 years of exposure to concentrates compared to patients with type 1, where all cases developed after 12 years.

Other groups have suggested severe liver disease occurs in relation to type 1 infections,²² especially type 1b.²⁰ However, it should be appreciated that the natural history of HCV infection in hemophiliacs may differ from that of other groups because there are important differences with respect to viral load, mixed HCV genotype infections, and immune suppression associated with clotting factor concentrates. The emergence of a relationship between a particular HCV geno-

type and progressive liver disease is highly complex and undoubtedly reflects a number of interrelated factors affecting virus-host interactions.

There is increasing evidence that the natural history of HCV-related liver disease may be greatly influenced by HCV genotype. The global distribution of a large number of genotypes and subtypes of HCV has resulted in a complex pattern of HCV infection in hemophiliacs treated with pooled plasma-derived products. In view of the growing evidence that the eventual clinical outcome, including interferon responsiveness, of HCV infection may be influenced by HCV genotype, it seems clear that this will have considerable impact on the natural history of HCV-related chronic liver disease in hemophiliacs in different parts of the world.

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